Journal of Radiation Research and Applied Sciences xxx (2016) 1–10

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Journal of Radiation Research and Applied Sciences

journal homepage: http://www.elsevier.com/locate/jrras

Optimization of antibacterial activity of *Eucalyptus tereticornis* leaf extracts against *Escherichia coli* through response surface methodology

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ARTICLE INFO

Article history:
Received 21 February 2016
Received in revised form
2 May 2016
Accepted 5 May 2016
Available online xxx

Keywords:
Medicinal plants
Eucalyptus tereticornis
Leaf extract
Antibacterial activity
Response surface methodology

ABSTRACT

Medicinal plants are the important source of potentially useful chemotherapeutic agents which have made enormous contributions to human health and well-being. The methanolic extract of leaves of *Eucalyptus tereticornis* for antibacterial activity against the bacterial strain *Escherichia coli* was investigated. Response surface methodology (RSM) using central composite design (CCD) was adopted to optimize the effect of process variables. The optimum conditions for maximum antibacterial activity (zones of inhibition, mm) were found out to be *E. tereticornis* extract i.e., Limonene, 3 μL; 1, 8-Cineole, 11 μL; Terpinen-4-ol, 15 μL; pH, 7.0 and temperature, 40 °C. The results suggest that the organic (methanol) extract of *E. tereticornis* could be a possible source to obtain new and effective herbal medicines to combat infections caused by multi-drug resistant microbial strains from community and hospital settings.

1. Introduction

The development of multiple drug resistant pathogens has diverted the attention of scientific community to search out new compounds with strong antimicrobial potential (Ashraf, Sarfraz, Rashid, & Shahid, 2015; Fazal & Rauf, 2015). Reports have shown that about 50,000 people die in the world per day due to infectious diseases mainly caused by *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. More than 80% of the world's population in the developing countries like Pakistan relies on plant-derived antimicrobial medicines to ensure safety of the treatment and minimize the risk of antimicrobial drug resistance in bacteria (Ahmad & Beg, 2001). A large number of studies have confirmed the potential efficacy of plant extracts against various bacterial and fungal pathogens. The broad range of microbial susceptibilities indicates the effectiveness of plant

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Peer review under responsibility of The Egyptian Society of Radiation Sciences and Applications.
http://dx.doi.org/10.1016/j.jrras.2016.05.001

Please cite this article in press as: Ammer, M. R., et al., Optimization of antibacterial activity of *Eucalyptus tereticornis* leaf extracts against *Escherichia coli* through response surface methodology, Journal of Radiation Research and Applied Sciences (2016), http://dx.doi.org/10.1016/j.jrras.2016.05.001

extracts for medicinal purposes as well as food additives to prevent spoilage. However, studies particularly on toxicity evaluation are needed to assess the suitability of these extracts for multipurpose functional uses (Fawad, Myaddad-ur-Rehamn, & Khan, 2012). According to World Health Organization (WHO), about 20,000 plant species have been reported to shown therapeutic potential (Ashraf et al., 2015), that may be attributed to the bioactive compounds synthesized during the secondary metabolism of plants (Fazal & Rauf, 2015).

Eucalyptus is a diverse genus of trees belonging to the *Myrtaceae* family and comprises about 800 species (Ogunwande, Olawore, Adeleke, Ekundayo, & Koenig, 2003). More than 300 species of this genus have volatile oils in their leaves; nevertheless less than 20 of these have yet been exploited commercially for the manufacturing of essential oils rich in 1, 8-cineole that used in the pharmaceutical and cosmetic industries (Brown, Del Pino, & Krohne, 2002). Leaf extracts of *Eucalyptus* have been recognized as natural food preservatives (Takahashi, Nagayama, & Mori, 2004), and have also been accepted as potential remedies for the treatment of many infectious diseases (Bello, Olabanji, Ibrahim, Yekeen, & Oboh, 2013; Tepe et al., 2004). Though, studies of its activity against pathogenic microorganisms are scarce (Bonello et al., 2010), many authors have reported the chemical composition, antioxidant and antimicrobial activities of *Eucalyptus* species (Sartorelli, Marquioreto, Amaral-Baroli, Lima, & Moreno, 2007). However, geographical distribution and species variation greatly affect these properties which require extensive studies to explore the potential of this plant.

Reducing the costs and making the processes economically viable by optimizing the operational key parameters is the ultimate objective of basic research for industrial applications (Asgher, Yasmeen, & Iqbal, 2014). Response Surface Methodology (RSM) is a statistical approach that widely used for developing, improving, and optimizing processes (Demarque et al., 2015; Hayes et al., 2005), and it determinates more than one factors at a time at different levels providing optimization results in a true sense (Rengadurai, Preetha, & Viruthagiri, 2012). Recently, it has been successfully applied in other scientific fields such as biology, medicine, and economy for optimization purposes (Curiel et al., 2004; Demarque et al., 2015). The objective of the current study was to evaluate the *in-vitro* antibacterial activity of methanolic extract of leaves of *Eucalyptus tereticornis* using RSM.

2. Materials and methods

2.1. Collection of plant material

Fresh leaves of *E. tereticornis* were collected from "The University of Lahore, Pakistan". The leaves were thoroughly washed under running tap water and allowed to dry at room temperature for 2 days. The dried material was crushed into fine powder in an electric grinder and stored in airtight bottles.

2.2. Extraction

The 50 g of dried leaves powder was thoroughly mixed with 200 mL methanol in triplicate conical flasks. The flasks containing extract were heated on boiling water bath for 1 h, wrapped with aluminum foil and kept at room temperature for 5 days with occasional shaking. After stipulated time, the liquid extract of flasks was transferred to falcon tubes and subjected to centrifugation at 5000 rpm for 10 min. The clear supernatants from the falcon tubes were shifted to pre-weighed beakers, and allowed to evaporate the solvent in hot water bath to get dried methanol free extract of *E. tereticornis*.

2.3. Test microorganism and inoculum preparation

The bacterial strain *E. coli* was obtained from the Department of Microbiology, Pakistan Council of Scientific & Industrial Research (PCSIR), Laboratories Complex Lahore, and maintained on Nutrient agar slants (Oxide) at 4 °C. A loop-full culture of *E. coli* was inoculated into nutrient broth under sterile conditions, and incubated at 37 °C for 24 h in rotary shaker. McFarland standard was used as a reference to adjust the turbidity of bacterial suspension in the range of 1×10^8 bacterial cells/mL, and was maintained throughout the study (Kachhiya, 2008; Perilla, Ajello, Bopp, Elliott, & Facklam, 2003).

2.4. Antimicrobial assay

The agar disc diffusion method was used to evaluate the antibacterial potentiality of *E. tereticornis* plant extract against *E. coli* using Mueller Hinton agar medium (Tenover et al., 1998). A 100 μL of freshly prepared inoculum (containing 1×10^8 bacterial cells/mL as per McFarland standard) was spread onto the surface of sterile Mueller Hinton agar using sterilized glass spreader. Filter paper discs of approximately 6 mm in diameter were soaked with 15 μL of the plant extract and placed in the agar plates. Each disc was pressed down to ensure complete contact with the agar surface and distributed evenly so that they are no closer than 24 mm from each other, center to center. The plates were incubated aerobically at 37 °C for 16–18 h. After specified time, each plate was examined, and resulting zones of inhibition (mm) were measured. The negative and positive controls were also prepared in parallel with only dimethyl sulfoxide [(DMSO) 4.0%, *v/v*] and Ciprofloxacin (50 μg/mL), respectively. Strict aseptic conditions were followed for carrying out the entire microbial assay.

2.5. Experimental design and statistical analysis

For optimization of antimicrobial activity of *E. tereticornis* leaf extracts, an experimental design containing five factors at four different levels was applied in triplicate under RSM with central composite design (CCD). Four different concentrations of *E. tereticornis* extract i.e., Limonene (3, 4, 5, 6 μL), 1, 8-Cineole (8, 9.5, 11, 12.5 μL), Terpinen-4-ol (14, 15, 16, 17 μL), and pH (5, 6, 7, 8) and temperature (35, 37.5, 40, 42.5 °C) were selected as independent variables and designated as A, B, C, D and E, respectively (Table 1). A total number of 25 runs (Table 2) were carried out to estimate the coefficients for the optimization of antibacterial activity of the selected plant. Upon completion of experiments, the data was subjected to Analysis of Variance (ANOVA) and three dimensional response surface plots were constructed using Design of Experts software (DOE version, 7.1.3, STAT-EASE Inc., Minneapolis, USA), that represent the individual and interactive effects of test variables on the response (antimicrobial activity). The quality of the fit of this model was expressed by the

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